FEBS Letters 423 (1998) 376–380 FEBS 19893

MK-801 blocks monoamine transporters expressed in HEK cells

Mitsuhiro Nishimura[a,*], Kohji Sato[b], Tomoya Okada[a], Patrick Schloss[c], Shoichi Shimada[d], Masaya Tohyama[a]

[a]*Department of Anatomy and Neuroscience, Osaka University Medical School, Yamadaoka 2-2, Suita, Osaka 565, Japan*
[b]*Department of Anatomy, Ehime University School of Medicine, Shigenobu, Ehime 791-02, Japan*
[c]*Biochemistry Department, University of Dublin, Trinity College, Dublin 2, Ireland*
[d]*Department of Anatomy, Nagoya City University Medical School Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467, Japan*

Received 5 January 1998; revised version received 27 January 1998

Abstract (+)-MK-801 is known to be a specific non-competitive antagonist of *N*-methyl-D-aspartate (NMDA) receptors. However, besides having an anticonvulsant effect, this compound possesses a central sympathomimetic effect and an anxiolytic-like action, raising the possibility that (+)-MK-801 might affect monoamine uptake systems. To elucidate this possibility, we investigated the effects of (+)-MK-801 on monoamine transporters expressed in HEK cells. (+)-MK-801 significantly inhibited the uptake of all three monoamine transporters in a dose-dependent manner and the inhibitions were competitive with respect to monoamines. The K_i values of (+)-MK-801 on the norepinephrine, dopamine and serotonin transporters were 3.2 μM, 40 μM and 43 μM, respectively. In addition, (−)-MK-801, a less potent antagonist of NMDA receptors, also inhibited monoamine transporters with a similar potency as that of (+)-MK-801. These results clearly indicate that MK-801, a non-competitive antagonist of NMDA receptors, competitively inhibits monoamine transporters without stereoselectivity.

Key words: MK-801; Norepinephrine transporter; Dopamine transporter; Serotonin transporter; *N*-Methyl-D-aspartate receptor; Human embryonic kidney cell

1. Introduction

(+)-MK-801 ((+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine maleate) is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist binding the phencyclidine (PCP) binding site of the receptors [1]. Like phencyclidine, (+)-MK-801 is also known to possess central sympathomimetic and psychotomimetic effects as well as a potent anticonvulsant effect [2–4]. These symptoms raise the possibility that (+)-MK-801 might affect monoaminergic neurotransmission. In fact, many studies have reported that monoamine systems are potentiated after administration of (+)-MK-801 [5–8]. Furthermore, some studies have shown using slices or synaptosomes that (+)-MK-801 inhibits monoamine uptake [9–11]. These findings strongly suggest that (+)-MK-801 inhibits transporter-mediated monoamine uptake, leading to the potentiation of monoaminergic neurotransmission. However, the exact relationship between (+)-MK-801 and monoamine transporters has not been well delineated.

*Corresponding author. Fax: (81) (6) 879-3229.
E-mail: mnishimu@anat2.med.osaka-u.ac.jp

Abbreviations: MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine maleate; CPP, 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; HEK cell, human embryonic kidney cell

Recently, monoamine (norepinephrine, dopamine and serotonin) transporters, responsible for the high-affinity monoamine uptake systems, have been cloned [12–18]. These transporters are widely expressed in monoaminergic neurons in the central nervous system and have been shown to be of great importance in many physiological and psychological phenomena. The discovery of these genes also enabled us to directly study the effects of (+)-MK-801 on each monoamine transporter. Thus, we constructed cell lines which stably express monoamine transporters, and investigated the effects of (+)-MK-801 on them. In the present study, we show that MK-801, a non-competitive antagonist of NMDA receptors, competitively inhibits monoamine transporters without stereoselectivity.

2. Materials and methods

2.1. Materials

(+)-MK-801, (−)-MK-801 and 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) were purchased from Research Biochemicals International (Massachusetts, USA). [^{3}H]Norepinephrine, [^{3}H]dopamine, [^{3}H]serotonin and [^{14}C]glutamate with specific activities of 15.0, 21.5, 24.4 and 261.6 Ci/mmol were purchased from New England Nuclear, Boston, MA.

2.2. Construction of HEK cells stably expressing transporters

Human embryonic kidney cells (HEK 293 cells; ATCC CRL1573) were transfected using the Chen-Okayama method with the human norepinephrine transporter (NET), rat dopamine transporter (DAT), rat serotonin transporter (SERT) and bovine glutamate/aspartate transporter (GLAST) cDNAs subcloned into the eukaryotic expression vector pBK/CMV (Stratagene), which contains the gene for geneticin resistance. The human norepinephrine transporter cDNA clone (pNET) was a kind gift of Dr. Susan Amara (Oregon Health Sciences University, USA). The rat cDNAs for the dopamine transporter and serotonin transporter were cloned by Shimada and Schloss, respectively. 24 h after the transformation, geneticin (1 mg/ml) was added into DMEM/10% FCS and selection was done for 4 weeks. Cells which obtained geneticin resistance were used for further experiments. We designated these cells HEK-NET, HEK-DAT, HEK-SERT and HEK-GLAST cells.

2.3. Uptake assay

For uptake measurements, HEK-NET, HEK-DAT, HEK-SERT and HEK-GLAST cells were plated into 24-well dishes. After these cells had reached confluence, the culture medium was removed and the cells were washed once with 0.2 ml transport buffer (TB: 125 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.5). After washing, 0.2 ml TB containing [^{3}H]norepinephrine, [^{3}H]dopamine, [^{3}H]serotonin or [^{14}C]glutamate was applied into each well to yield final concentrations of 133.4 nM, 93.0 nM, 80.2 nM and 1.53 μM, respectively. Following incubation at room temperature for 6 min, the solution was removed by suction and the cells were washed twice with TB and then extracted with 0.4 ml of 10% (w/v) sodium dodecyl sulfate. Radioactivity was determined by scintillation counting using a Beckman LS600011C scintillation counter.

PII S0014-5793(98)00126-4

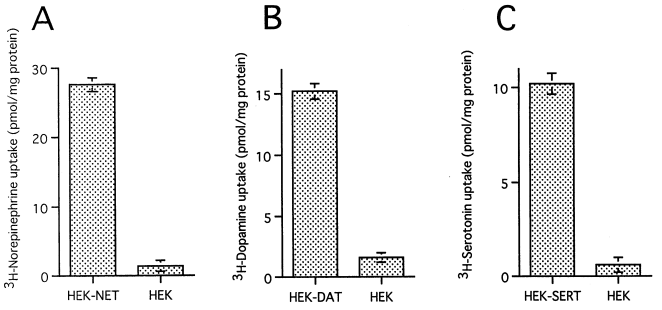

Fig. 1. Ligand uptake into HEK-NET, DAT, SERT cells and HEK cells (control). The cells were incubated with transport buffers containing 133.4 nM [^{3}H]norepinephrine, 93.0 nM [^{3}H]dopamine or 80.2 nM [^{3}H]serotonin. Uptake was determined after 6 min. Each point represents the mean ± S.D. of two experiments run in quadruplicate.

The specific [^{3}H]monoamine and [^{14}C]glutamate uptakes were determined as the difference between the uptake values measured in HEK-NET, DAT, SERT or GLAST and those in untransfected HEK. Three independent experiments each in quadruplicate were performed. Inhibition curves were fitted (Cricket Graph III, Computer Associates International Inc., USA) to determine the IC_{50} and Hill coefficient. IC_{50} and Hill coefficient were determined using non-linear iterative curve fitting to a sigmoid function: $V = 100 \times I^h/(I^h + C^h)$, where V is % control of uptake, I is the IC_{50} value, h is the Hill coefficient, and C is the concentration of MK-801. Then we converted IC_{50} values to K_i values using the Cheng and Prusoff equation, $K_i = IC_{50}/(1+[L]/K_m)$ where [L] is the radioligand concentration of norepinephrine, dopamine or serotonin. Protein concentrations were determined by the Detergent Compatible Protein Assay (Bio-Rad), using bovine serum albumin as a standard.

3. Results

3.1. Assessment of HEK cells expressing monoamine transporters

To assess the ability of HEK cells expressing monoamine transporters (HEK-NET, HEK-DAT and HEK-SERT cells) to take up the corresponding molecules, we performed uptake assays. After 6 min incubation with 133.4 nM [^{3}H]norepinephrine, 93.0 nM [^{3}H]dopamine or 80.2 nM [^{3}H]serotonin, HEK-NET, HEK-DAT and HEK-SERT cells took up 27.6 ± 1.0, 15.2 ± 0.64, and 10.2 ± 0.56 pmol/mg protein of the corresponding molecules, respectively (Fig. 1A–C). These values were 19.7, 9.5 and 17 times higher than those of control HEK cells respectively, showing that these three cell lines express the corresponding functional transporters.

Uptake was linear with respect to time at least until 8 min for each transporter (data not shown). Thus, in the following experiments, uptake was routinely determined after 6 min at room temperature. Competition studies with increased concentration of unlabeled substrate revealed that K_m values for [^{3}H]norepinephrine, [^{3}H]dopamine or [^{3}H]serotonin were about 864 nM, 1406 nM, 1170 nM, respectively.

3.2. Effect of (+)-MK-801 on monoamine transporters

To investigate the effect of (+)-MK-801 on the norepinephrine transporter, we added various concentrations of (+)-MK-801 to the uptake buffer (the transport buffer containing 133.4 nM [^{3}H]norepinephrine) and performed inhibition assays using HEK-NET cells. The uptake of tracer was hardly inhibited at 100 nM. However, above 100 nM, (+)-MK-801 significantly inhibited the uptake dose-dependently (Fig. 2A). The IC_{50} value was 3.7 ± 1.2 μM, and the Hill coefficient was 0.98 ± 0.02 ($n = 3$). To exclude the possibility that the inhibition is mediated by NMDA receptors, we performed the same experiments with CPP, a competitive NMDA receptor antagonist. However, CPP did not affect norepinephrine uptake (Fig. 2A).

We also investigated using HEK-DAT cells the effect of (+)-MK-801 on the dopamine transporter. (+)-MK-801 inhibited the dopamine transporter above 1 μM. At 1 mM, (+)-MK-801 inhibited it almost completely (Fig. 2B). The IC_{50} value was 435.8 μM and the Hill coefficient was 0.97 ± 0.02 ($n = 3$). In contrast, CPP did not inhibit dopamine uptake (Fig. 2B).

(+)-MK-801 also inhibited serotonin uptake mediated by the serotonin transporter above 1 μM (Fig. 2C). At 1 mM, (+)-MK-801 inhibited it almost completely (Fig. 2C). The IC_{50} value was 475.8 μM and the Hill coefficient was 0.95 ± 0.03 ($n = 3$). CPP did not affect serotonin uptake (Fig. 2C).

3.3. Competition experiments

To elucidate whether (+)-MK-801 inhibits monoamine transporters competitively or non-competitively, we measured uptake velocities under various concentrations of substrate in the presence or absence of (+)-MK-801 for each transporter. Lineweaver-Burk double reciprocal plot analyses showed that, for each transporter, two lines intercepted on the vertical axis, clearly indicating that the (+)-MK-801-induced inhibition was

Table 1
Effects of (+)- and (−)-MK-801 on monoamine transporters expressed in HEK cells

	(+)-MK-801		(−)-MK-801	
	K_i (μM)	Hill coefficient	K_i (μM)	Hill coefficient
NET	3.1 ± 1.1	0.98 ± 0.02	3.7 ± 3.2	0.97 ± 0.03
DAT	40 ± 5.4	0.97 ± 0.02	40 ± 5.7	0.96 ± 0.02
SERT	43 ± 5.4	0.95 ± 0.03	47 ± 8.8	0.94 ± 0.04

Each value represents the mean ± S.E.M. of three independent experiments performed in quadruplicate.

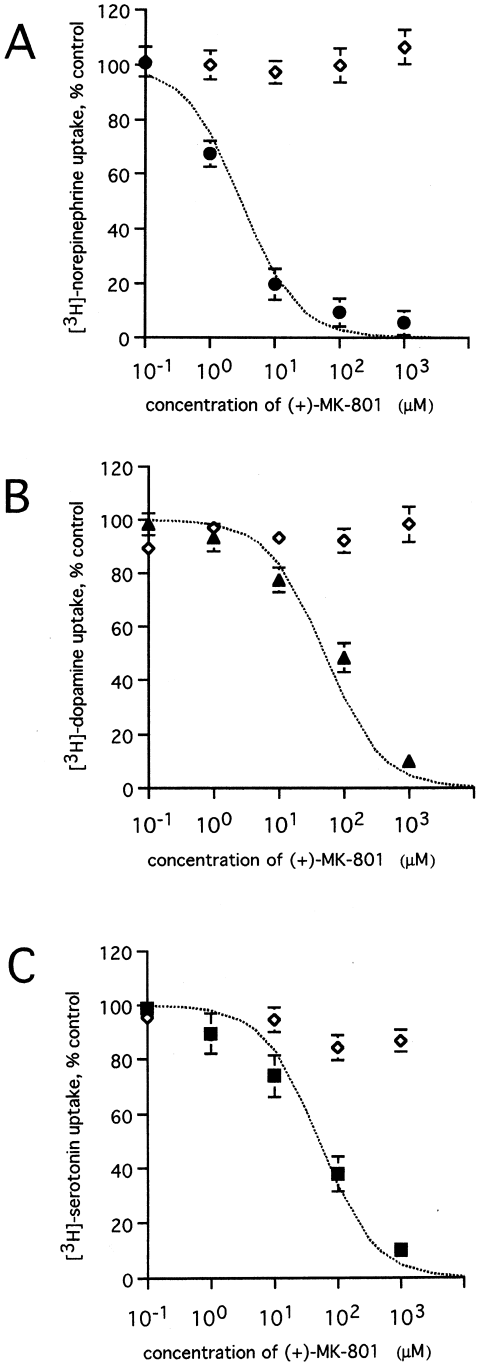

Fig. 2. Effects of (+)-MK-801 and CPP on the norepinephrine transporter (A), dopamine transporter (B) and serotonin transporter (C). HEK-NET, HEK-DAT or HEK-SERT cells were incubated with transport buffers containing 133.4 nM [^{3}H]norepinephrine, 93.0 nM [^{3}H]dopamine or 80.2 nM [^{3}H]serotonin and various concentrations of MK-801 (filled symbols) or CPP (open diamonds). Uptake was determined after 6 min. Each point represents the mean ± S.E.M. of three experiments run in quadruplicate.

competitive for all three monoamine transporters (Fig. 3). Thus, we converted the IC_{50} values to K_i values using the Cheng and Prusoff equation. K_i values of (+)-MK-801 for the norepinephrine, dopamine and serotonin transporters were 3.2 ± 1.1 μM, 40 ± 5.4 μM, and 43 ± 5.4 μM, respectively (Table 1).

3.4. Effect of (+)-MK-801 on a glutamate transporter

To elucidate whether (+)-MK-801 specifically inhibits monoamine transporters, we investigated the effects of (+)-MK-801 on bovine glutamate/aspartate transporter (GLAST) stably expressed in HEK cells. After 6 min incubation with 1.53 μM [^{14}C]glutamate, HEK-GLAST cells took up 590 ± 45.1 pmol/mg protein of [^{14}C]glutamate, and this value was significantly higher than that of control HEK cells, which was 163 ± 13.0 pmol/mg protein of [^{14}C]glutamate ($P < 0.01$). Competition studies with increased unlabeled substrate revealed its K_m value to be 70 μM. We compared the inhibitory effect of a high concentration of (+)-MK-801 on the glutamate/aspartate transporter to those on monoamine transport-

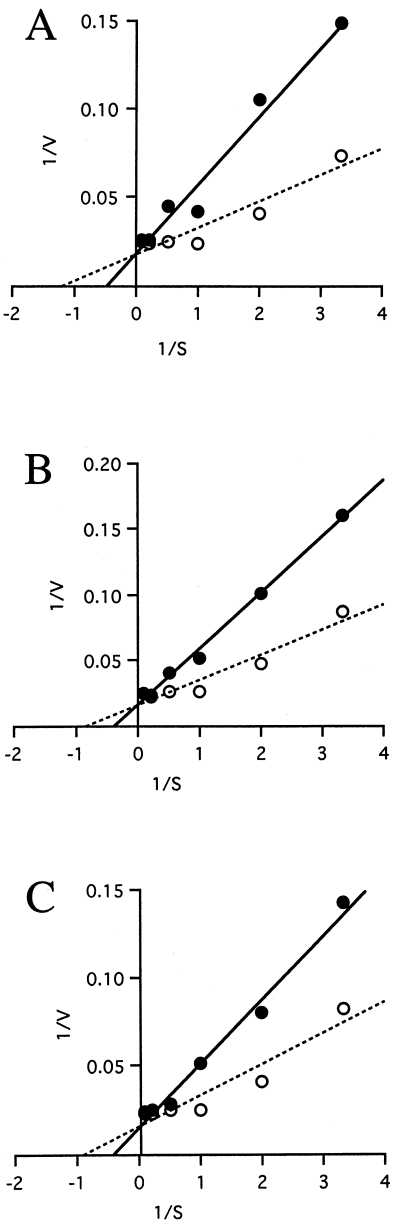

Fig. 3. Lineweaver-Burk plots of (+)-MK-801 inhibition on the norepinephrine transporter (A), dopamine transporter (B), and serotonin transporter (C). Uptake velocities (V [pmol/mg protein/min]) were measured for 6 min on various substrate concentrations (S [μM]) in the presence (●, solid line) and the absence (○, broken line) of (+)-MK-801 (3.2 μM in A, 40 μM in B, 43 μM in C).

ers. 1 mM (+)-MK-801 inhibited the glutamate/aspartate transporter only 8.2 ± 2.4%, while the same dose of (+)-MK-801 inhibited monoamine transporters almost completely (94.5 ± 4.4% on the norepinephrine transporter, 89.8 ± 3.6% on the dopamine transporter, and 89.4 ± 1.4% on the serotonin transporter), indicating that (+)-MK-801 preferentially inhibits monoamine transporters.

3.5. Effect of (−)-MK-801 on monoamine transporters

To elucidate whether the inhibition is stereoselective or not, we studied the effect of (−)-MK-801, a less active form of the NMDA receptor, on monoamine transporters. Inhibition analysis revealed that K_i values of (−)-MK-801 for norepinephrine, dopamine and serotonin transporter were 3.7 ± 3.2 μM, 40 ± 5.7 μM, and 47 ± 8.8 μM, respectively (Table 1), indicating that there was no significant difference in the potency of inhibiting monoamine uptake between the two optical isomers.

4. Discussion

(+)-MK-801 is widely used as a non-competitive NMDA receptor antagonist. Besides as an anticonvulsant, it is also known to have sympathomimetic and psychotomimetic properties [2–4]. This suggests that monoaminergic systems are involved in the pharmacological mechanism of (+)-MK-801. In fact, many lines of evidence indicate that monoaminergic systems are potentiated after (+)-MK-801 administration [5–8]. Although this potentiation has been mainly thought to be mediated by the inhibition of the NMDA receptor itself, some studies have shown using slices or synaptosomes that (+)-MK-801 directly inhibits monoamine uptake [9–11]. According to these studies, (+)-MK-801 blocks most potently [^{3}H]norepinephrine uptake (IC_{50} about 2 μM) and [^{3}H]dopamine uptake with relatively low potency (IC_{50} about 100 μM). Our present data coincide well with these values, supporting the possibility that the monoamine uptake systems inhibited by (+)-MK-801 in vivo are exactly monoamine transporters. In addition, to exclude the possibility that the (+)-MK-801-induced inhibition is mediated by blocking NMDA receptors expressed in HEK cells, we performed the same experiments using CPP, a competitive NMDA receptor antagonist. However, even 1 mM CPP showed no effect on the uptake, indicating that (+)-MK-801 directly affects monoamine transporters.

Like PCP, (+)-MK-801 has been known to have two binding sites in the brain [19,20]. Site 1 is associated with NMDA receptors and site 2 has been postulated to be monoamine uptake systems. The affinity of (+)-MK-801 on site 2 are reported to be about 5–10 μM. These values coincide well with the K_i value of the norepinephrine transporter obtained in the present study (3.2 μM), suggesting that site 2 might be the norepinephrine transporter. Additionally, most compounds binding the PCP site of NMDA receptors such as PCP and ketamine have been reported to bind monoamine transporters as well [19,20]. It is, thus, intriguing to compare the pharmacological properties of these two binding sites for MK-801. A mutation analysis has clearly shown that the binding site for MK-801 on NMDA receptor subunit 1 requires an interplay of residues from its transmembrane 2 and 3 segments, which are situated in the center of the ionic pore [21]. In addition, the authors have speculated that hydrogen bonding between asparagine at position 582 and imine (-NH) on MK-801 is important for the binding. Furthermore, Leeson et al. reported that the MK-801 recognition site on the NMDA receptor ion channel requires size-limited hydrophobic binding of both aromatic rings, and strictly directional hydrogen bond donation from protonated quaternary nitrogen atom [22]. These findings suggest that the binding site of the NMDA receptor for MK-801 is highly selective. In fact, the site shows strict stereoselectivity for MK-801 [1]. Wong et al. have reported that (−)-MK-801 is one-seventh as potent as (+)-MK-801. In contrast, as we present in this study, the binding sites on monoamine transporters show no stereoselectivity for MK-801. The monoamines, substrates of monoamine transporters, share structural similarity with one of the aromatic rings and the nitrogen atom of MK-801. Thus, we speculate that MK-801 would bind monoamine transporters only with one of the benzene rings and the imine residue, causing the relatively weak affinity for monoamine transporters.

Since MK-801 is a 'non-competitive' antagonist of NMDA receptors, its binding site and the glutamate binding site on the receptor complex are completely different. On the other hand, we found that the inhibitory effects of MK-801 for monoamine transporters are competitive with respect to monoamines, indicating that the MK-801 binding sites on monoamine transporters overlap the monoamine binding sites on them. It might be of interest to find where these sites are located in each protein, and to investigate the structural relationship among them.

(+)-MK-801 is normally used at doses of 0.1–0.5 mg/kg when intraperitoneally injected into rats [9–11]. Vezzani et al. reported that these doses give maximal concentrations of about 0.2–1.0 μM in the brain [23]. These concentrations seem enough to affect at least the norepinephrine transporter, because (+)-MK-801 gives almost 30% inhibition at 1 μM (Fig. 2A). In addition, if the concentration of (+)-MK-801 in the synaptic cleft is higher than this estimation, it might be possible that the dopamine and serotonin transporters are also affected. Thus, when (+)-MK-801 is used as a specific antagonist of the NMDA receptor, the possibility that (+)-MK-801 also affects monoamine transporters should be cautiously considered.

Acknowledgements: We thank Dr. Susan G. Amara for supplying norepinephrine transporter cDNA. This work was supported in part by the Ministry of Education, Science and Culture of Japan.

References

[1] Wong, E.H., Kemp, J.A., Preistley, T., Knight, A.R., Woodruff, G.N. and Iversen, L.L. (1986) Proc. Natl. Acad. Sci. USA 83, 7104–7108.
[2] Clineschmidt, B.V., Martin, G.E., Bunting, P.R. and Papp, N.L. (1982) Drug Dev. Res. 2, 123–134.
[3] Clineschmidt, B.V., Martin, G.E. and Bunting, P.R. (1982) Drug Dev. Res. 2, 135–145.
[4] Rogawske, M.A. and Porter, R.J. (1990) Pharmacol. Rev. 42, 259–266.
[5] Hiramatsu, M., Cho, A.K. and Nabeshima, T. (1989) Eur. J. Pharmacol. 166, 359–366.
[6] Löscher, W. and Hönack, D. (1992) Eur. J. Pharmacol. 215, 199–208.
[7] Whitton, P.S., Biggs, C.S., Pearce, B.R. and Fowler, L.J. (1992) J. Neurochem. 58, 1573–1575.
[8] Whitton, P.S., Biggs, C.S., Pearce, B.R. and Fowler, L.J. (1992) Neurosci. Lett. 142, 5–8.

[9] Pubill, D., Canudas, A.M., Gasulla, D., Pallas, M., Escubedo, E. and Camarasa, J. (1996) Eur. J. Pharmacol. 303, 171–175.
[10] Snell, L.D., Yi, S.J. and Johnson, K.M. (1988) Eur. J. Pharmacol. 145, 223–226.
[11] Clarke, P.B. and Reuben, M. (1995) Br. J. Pharmacol. 114, 315–322.
[12] Pacholczyk, T., Blakely, R.D. and Amara, S.G. (1991) Nature 350, 350–354.
[13] Shimada, S., Kitayama, S., Lin, C.L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M. and Uhl, G. (1991) Science 254, 576–578.
[14] Kilty, J.E., Lorang, D. and Amara, S.G. (1991) Science 254, 578–579.
[15] Giros, B., el Mestikawy, S., Bertrand, L. and Caron, M.G. (1991) FEBS Lett. 295, 149–154.
[16] Usdin, T.B., Mezey, E., Chen, C., Brownstein, M.J. and Hoffman, B.J. (1991) Proc. Natl. Acad. Sci. USA 88, 11168–11171.
[17] Hoffmann, B.J., Merzey, E. and Brownstein, M.J. (1991) Science 254, 579–580.
[18] Blakely, R.D., Berson, H.E., Fremeau, R.T., Caron, M.G., Peek, M.M., Prince, H.K. and Bradley, C.C. (1991) Nature 354, 66–70.
[19] Rothman, R.B., Reid, A.A., Monn, J.A., Jacobson, A.E. and Rice, K.C. (1989) Mol. Pharmacol. 36, 887–896.
[20] Akunne, H.C., Reid, A.A., Thurkauf, A., Jacobson, A.E., Costa, B.R., Rice, K.C., Heyes, M.P. and Rothman, R.B. (1991) Synapse 8, 289–300.
[21] Ferrer-Montiel, A.V., Sun, W. and Montal, M. (1995) Proc. Natl. Acad. Sci. USA 92, 8021–8025.
[22] Leeson, P.D., James, R.W., Carling, R.W., Wong, E.H. and Baker, R. (1990) Prog. Clin. Biol. Res. 361, 513–518.
[23] Vezzani, A., Serafini, R., Stasi, M.A., Caccia, S., Conti, I., Tridico, R.V. and Samanin, R. (1988) J. Pharmacol. Exp. Ther. 249, 278–283.